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Toward Quality Assurance for Metaphase FISH: A Multi-Center Experience

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Although fluorescent in situ hybridization (FISH) is rapidly becoming a part of clinical cytogenetics, no organization sponsors multi-center determinations of the efficacy of probes. We report on 23 laboratories that volunteered to provide slides and to use a probe for SNRPN and a control locus. Experiences with FISH for these laboratories during 1994 ranged from 0 to 645 utilizations (median = 84) involving blood, amniotic fluid and bone marrow. In an initial study of hybridization efficiency, the median percentage of metaphases from normal individuals showing two SNRPN and 2 control signals for slides prepared at each site was 97.0 (range = 74–100); for slides prepared by a central laboratory, it was 97.8 (range = 81.6–100). In a subsequent blind study, each laboratory attempted to score 5 metaphases from each of 23 specimens [8 with del(15)(q11.2→q12) and 15 with normal 15 chromosomes]. Of 529 challenges, the correct SNRPN pattern was found in 5 of 5 metaphases in 457 (86%) and in 4 of 5 in 33 (6%). Ambiguous, incomplete or no results were reported for 32 (6%) challenges. Seven (1%) diagnostic errors were made including 6 false positives and 1 false negative: 1 laboratory made 3 errors, 1 made 2, and 2 made 1 each. Most errors and inconsistencies seemed due to inexperience with FISH. The working time to process and analyze slides singly averaged 49.5 minutes; slides processed in batches of 4 and analyzed singly required 36.9 minutes. We conclude that proficiency testing for FISH using an extensive array of challenges is possible and that multiple centers can collaborate to test probes and to evaluate costs.

KEY WORDS: mosaicism, proficiency testing, Prader-Willi syndrome, SNRPN probe, metaphase FISH, quality assurance, hybridization efficiency, workload

INTRODUCTION

Fluorescence in situ hybridization (FISH) is rapidly becoming part of clinical practice in cytogenetics. Fluorescent DNA probes are available as chromosome-specific paints (CSP), for chromosome-specific alpha satellites (alphoids) and for certain locus-specific sites (cosmids). FISH is used to examine cells in interphase and metaphase, and can be applied to almost any type of tissue [Trask, 1991]. However, the reproducibility, normal range and accuracy of FISH in clinical practice has been established for only a few probes [Jenkins et al., 1992; Kibbelaar et al., 1993; Dewald et al.,

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1993a,b; Lu et al., 1994; Schad and Dewald, 1995]. Moreover, no probe for the nascent technology has been approved for diagnostic services by the Food and Drug Administration.

We are not aware of any group being organized to collect data from the same subjects to validate probes for FISH, to measure the effort (workload) associated with FISH, to test the efficacy of FISH in clinical service or to assess the accuracy of laboratories through proficiency testing. We organized 23 cytogenetic laboratories in the Great Lakes Regional Genetics Group (GLaRGG) to test the possibility of accomplishing all 4 of these goals. We report on a project involving laboratories that volunteered to provide slides and to use FISH with DNA probes for the small nuclear ribonucleoprotein polypeptide N (SNRPN) site in 15q11→13 [Ozcelik et al., 1992] and a control locus in 15q22 for the PML gene.

MATERIALS AND METHODS

Participants

Fifty-nine institutions providing clinical cytogenetic services within the states covered by GLaRGG (IL, IN, MI, MN, OH and WI) were contacted by letter about participating in a possible project with FISH. Thirty-three laboratories responded and 23 agreed to provide slides and to dedicate personnel for participation. Although self-selected, the participants represented a reasonable sample of cytogenetic laboratories in the United States.

Equipment, Experience and Organization

By questionnaire, participants were asked about the filters, light sources and wattages, and brands and models of the microscopes used for FISH. From this information, Oncor, Inc. provided each participant with reagents and probe from a single lot for detection with fluoroisothiocyanate (FITC) and propidium iodide (PI), with Rhodamine and 4′-6′-diamidine-2-phenylindole dihydrochloride (DAPI) or with FITC and DAPI. Each participant was also asked about the number of times CSP, alphoid and cosmid probes were used in 1994 for clinical evaluations of peripheral blood, amniotic fluid and bone marrow. Each application of a probe to a sample was counted as a separate utilization.

A general plan describing the course of the project was supplied to each participant. On specific dates, laboratories prepared slides and shipped them synchronously to one of the authors (RS) by overnight delivery. RS returned a set of coded slides to each participant by overnight delivery so that the age of the slides could be kept approximately constant for all sites. Special forms and instructions were created before each step of the project and were shipped to the participants at the appropriate times to help assure that data were collected and recorded uniformly. Results were summarized and distributed so that participants could know their own results and could compare them anonymously to those of other participants.

The cosmid probe for the SNRPN site was chosen because of its common utilization for studying chromosomes of patients with the Prader-Willi syndrome. The probe detects microscopic and submicroscopic deletions in 15q11.2→q12 [Ozcelik et al., 1992], but it does not detect genic or uniparental disomic causes of the syndrome.

Hybridization Efficiency

Hybridization efficiency of a probe in a FISH study is defined as the percentage of scorable metaphases with a number of fluorescent signals matching the cytogenetic standard. The hybridization efficiency of SNRPN in each laboratory was established with slides prepared by the participants themselves and with slides prepared by a central laboratory. Participants prepared a single slide using lymphocytes from a chromosomally normal individual. That slide and a partial karyotype of the chromosomes 15 were then shipped to RS. At the same time, a central laboratory prepared 23 slides using lymphocytes from a chromosomally normal individual and sent them and a partial karyotype of the chromosomes 15 to RS. The slide prepared by each laboratory was labeled *A* and the slides from the central laboratory were labeled *B*. Each *A* slide was then returned to the laboratory that made it along with a *B* slide.

Each participant was to examine the *A* and the *B* slide in the same way to collect 50 consecutive metaphases meeting the manufacturer's criteria for scoring SNRPN signals, i.e., only metaphases with two control signals could be scored. Participants were to record the number of scorable metaphases with 0, 1, 2 or >2 SNRPN signals. If unable to score 50 metaphases showing two control signals, participants were to examine and record as many scorable metaphases as possible. Additionally, participants were to count the number of metaphases with 0, 1 or ≥3 control signals encountered while examining each slide. These metaphases, not meeting the scoring criteria, were used to calculate the percentage of unacceptable metaphases. Results were recorded and returned for coding within 1 week.

Blind Study

Once studies of hybridization efficiency were completed, each participant provided slides with metaphases from the leukocytes of either a chromosomally normal individual or one known to have a nonmosaic del(15)(q11.2→q12) and the Prader-Willi syndrome. Each laboratory prepared a set of 23 unlabeled slides and sent them and a partial karyotype of the chromosomes 15 to RS. These slides were coded and each laboratory was shipped a set of the 23 types of specimens to study blindly with the SNRPN probe. Participants attempted to collect 5 consecutive metaphases from each slide showing 2 control signals and to record the number of these metaphases with 0, 1, 2 or >2 SNRPN signals. While selecting the 5 scorable metaphases, participants also recorded the number of metaphases with 0, 1 or ≥3 control signals. The results were returned to RS within 2 weeks for analysis.

Workload Recording

The form for recording information about the workload associated with FISH followed the manufacturer's instructions for using the SNRPN probe, but the form was not a substitute for those instructions. Twenty-

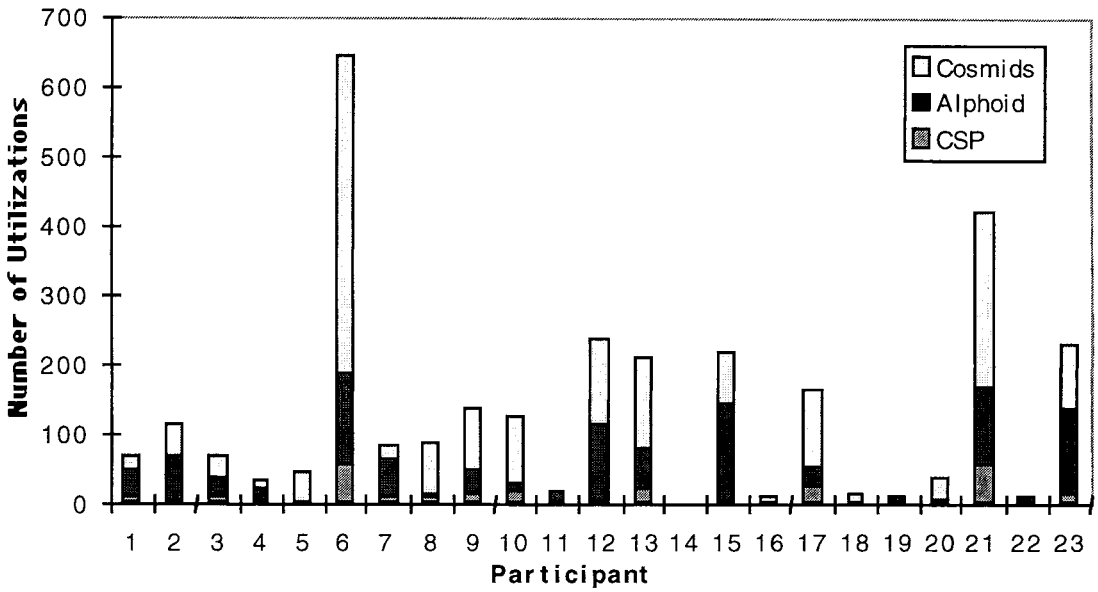

Fig. 1. Experience of each participating laboratory with FISH in 1994.

seven steps were delineated for timing. To give each site some preliminary experience before the actual timings, participants processed the slides in numerical order beginning with slide 1 and working through to slide 23. Timings to measure work in minutes and seconds were taken for slides 13 and 14 processed singly and for slides 15–18 and 19–22 processed in batches of 4. Participants provided timings for only successful hybridizations, but reported any rehybridizations required for slides 13 and 14.

RESULTS

Equipment

Nine laboratories used Zeiss microscopes, 7 used Olympus, 4 used Lietz and 3 used Nikon. Nineteen laboratories used 100-Watt mercury light sources, 3 used 50-Watt mercury light sources and one used both. Nineteen laboratories used the FITC/PI system for detecting fluorescence, 3 used rhodamine/DAPI and one used FITC/DAPI.

Experience With FISH

The total number of utilizations of probes with FISH for the 23 laboratories during 1994 was 2,974. The median number of utilizations was 84, but ranged from 0 to 645 (Fig. 1). Of the utilizations, 1,708 (57%) employed cosmids, 963 (32%) alphoids and 303 (10%) CSPs. Of the utilizations, 1,647 (55%) involved peripheral blood, 750 (25%) amniotic fluid and 577 (19%) bone marrow. FISH was used by 22 laboratories on peripheral blood, by 17 on amniotic fluid and by 17 on bone marrow (Table I). Only one laboratory did not use FISH for evaluations in 1994.

Hybridization Efficiency

Upon review by RS, none of the 23 partial karyotypes supplied by the participants for the chromosomes 15 on the *A* slides suggested a visible deletion in 15q11.2→q12. The partial karyotype supplied by a central laboratory for the chromosomes 15 on the *B* slides also did not suggest a deletion in 15q11.2→q12.

One laboratory could not perform the initial hybridizations of *A* or *B* and another laboratory only processed slide *B*. Seventeen of 21 laboratories found 50 metaphases on the *A* slides and 13 of 22 laboratories found 50 metaphases on the *B* slides. The 4 laboratories finding less than 50 metaphases on the *A* slides found 15, 43, 45, and 45 metaphases. The 9 laboratories finding less than 50 metaphases on the *B* slides found 13, 16, 37, 38, 39, 40, 43, 45, and 47 metaphases. No laboratory reported experiencing any problem with the slides.

Overall, participants examined 1,082 metaphases from the *A* slides and rejected 134 (12.4%) as unsuitable for scoring. Participants examined a total of 1,012 metaphases from the *B* slides and considered 94 (9.3%) unsuitable for scoring. For the *A* slides, the median percentage of unsuitable metaphases was 7.4 (range = 0–59.5%). For the *B* slides, the median percentage of unsuitable metaphases was 8.7 (range = 0–27.5%).

TABLE I. Experience of Participants With FISH by Tissue Type

Number of utilizations	Number of laboratories		
	PB[a]	AF[a]	BM[a]
0	1	6	6
1–20	6	10	10
21–50	6	2	5
51–100	5	1	1
101–300	3	4	0
301–650	2	0	1

[a] PB, peripheral blood; AF, amniotic fluid; BM, bone marrow.

The hybridization efficiency was calculated for the *A* and *B* slides by finding the mean percentage of all scorable metaphases with 2 SNRPN signals (Table II). For the *A* slides, the median hybridization efficiency was 97.0 (range = 74–100%) for 21 participants studying metaphases from 21 different patients. For the *B* slides, the median hybridization efficiency was 97.8 (range = 81.6–100%) for 22 participants studying metaphases from a single patient.

Blind Study

Seven distinctive styles of slides were submitted by the 23 participants. Four styles were unique to single participants. The 3 other styles were used by 7, 7 and 5 participants. Thus, in the blind study, only 4 participants could have recognized their own single submissions in the set of 23 slides each examined.

By conventional cytogenetic studies and by review of a partial karyotype from each of the chromosome 15 homologous pairs, 8 of the specimens were known to have a del(15)(q11.2→q12) and 15 were known to have normal chromosomes 15. In the blind study, the 23 participants attempted to score 5 metaphases from each of the 23 specimens for the presence or absence of SNRPN sites. Thus, as a group, the participants had a total of 529 challenges (23 participants times 23 challenges).

Participants examined 3,466 metaphases in the blind study. This total excludes the results from one laboratory that did not record "unacceptable" metaphases for any challenge and from single slides evaluated subjectively by 2 other laboratories. Of the metaphases 2,116 were observed on slides showing normal chromosomes 15 and 1,350 were observed on slides showing del(15)(q11.2→q12). Participants rejected 15.7% (median) of metaphases (range = 0–79 metaphases) examined on slides with normal metaphases; 33.9% (median) of metaphases (range = 0–111 metaphases) on slides with abnormal metaphases. The percentage of observations of single SNRPN signals on slides with normal metaphases and the percentage of observations of 2 SNRPN signals on slides with abnormal metaphases were both 3.3.

Of the 529 possible challenges, the correct SNRPN pattern was found in 5 of 5 metaphases in 457 (86.4%) and in 4 of 5 metaphases in 33 (6.2%; Table III). For 32 (6.0%) of the challenges, results were unreliable (2 metaphases with one SNRPN signal and 3 with 2 signals, or 3 metaphases with one SNRPN signal and 2 with 2 signals), incomplete (0–4 metaphases, but at least some metaphases examined), or no data (2 due to broken slides and 6 due to no metaphases observed). The number of participants finding 5 metaphases in 23, 22, 21, 20, 19, 18, 14, 12, and 10 of the challenges was 6, 6, 3, 2, 2, 1, 1, 1 and 1, respectively.

Seven diagnostic errors, including 6 false positives and one false negative, were reported. Thus, the overall percentage of diagnostic errors was 1.3 (7/529). Four participants made 1, 1, 2 and 3 diagnostic errors. These participants utilized FISH 210, 70, 7 and 15 times, respectively, in 1994. For the same period, the 2 participants reporting the greatest numbers of results with unreliable, incomplete or no data (9 of 23 and 7 of 23) utilized FISH 70 and 237 times, respectively. The participants agreed that the pattern of SNRPN signals matched the cytogenetic standard for each of the 23 specimens in no fewer than 17 of 23 examinations of metaphases from the same specimen. The correct pattern of fluorescence was reported for ≥18 of 23 challenges by 20 participants and for ≤14 of 23 challenges by 3 participants. None of the 23 types of specimens presented a consistent problem for participants.

Workload Recording

Each participant provided data from recording workload for slides processed one at a time and for slides processed in batches of 4 (Table IV). Some laboratories did not time all steps and some laboratories provided internally inconsistent timings. We used only data from the laboratories providing complete, internally consistent timings for the present report.

For 11 laboratories, the time to process one slide at a time averaged 19.9 minutes. When slides were processed in batches of 4 by 9 laboratories, the average time required per slide was 8.3 minutes. It took 11 laboratories on average 27.5 minutes to score 5 metaphases and to capture 2 documentary images. None of these 11 laboratories rehybridized either of the 2 slides processed singly. On average, it took 10 laboratories another 1.1 minutes to prepare solutions for each slide.

DISCUSSION

Organization

This project was designed to simulate FISH studies with SNRPN in clinical practice beginning with prepared slides and ending with the completion of analyses at the microscope (including capturing 2 documentary images of metaphases). The manufacturer's instructions for usage for the SNRPN probe proved functional. Some laboratories asked to tailor procedures to their own experiences. We discouraged deviations from the manufacturer's instructions to assure comparable data, especially data from the segment involving the recording of workload.

TABLE II. Percentages of Metaphases Scored for SNRPN Signals on *A* and *B* Slides

	A slides (21 patients)			*B* slides (22 slides, 1 patient)		
Signals	Median	Max	Min	Median	Max	Min
0	0.0	10.0	0.0	0.0	4.0	0.0
1	2.0	11.1	0.0	2.0	15.8	0.0
2	97.0	100.0	74.0	97.8	100.0	81.6
>2	0.0	6.0	0.0	0.0	4.0	0.0

TABLE III. Results of Blind Study Showing Normal and Abnormal Specimens Ordered Left to Right in Decreasing Levels of Consensus Based on 5 of 5 Metaphases

	Specimen number[a]																					
	Chromosomally normal													del(15)(q11.2q12)								
Metaphases	21	2	4	12	20	22	7	14	16	11	18	23	1	3	19	9	10	13	15	17	5	6	8
5 normal	23	22	22	22	22	22	21	21	21	20	19	19	18	18	18								[1]
5 abnormal									[2]		[2]	[1]				20	20	19	19	19	18	17	17
4 normal, 1 abnormal		1	1	1	1	1				3	1	2	3	4	5								
4 abnormal, 1 normal													[1]					1		1	2	4	2
Unreliable results							1	1								1	1	1	1		2	1	1
0–4							1	1				1				1	2	2	2	1	1		2
No data											1		1	1		1			1	2		1	

[a] Specimen number corresponds to the number assigned each participant in Figure 1. Boxes indicate diagnostic errors.

The project required each laboratory to complete the study to determine hybridization efficiency in 2 days and the blind study in 2 weeks. Requiring processing of slides from 23 specimens in a short time may have contributed to the outcomes for some participants. None, however, withdrew from the project when the exact calendar became available. All requests for accommodating the needs of specific participants were met within the framework of the project which ensured similar freshness for all slides and a timely return of data.

Hybridization Efficiency

The initial work in each laboratory provided all participants the opportunity to become familiar with a specific lot of the SNRPN probe and to review the manufacturer's instructions for utilization and scoring. Participants examined normal metaphases both on slides they had prepared themselves (*A*) and on slides prepared by a central laboratory (*B*). Overall, there was no notable difference between the numbers of metaphases scored or the percentages of metaphases rejected from the *A* (21 different subjects) and the *B* (22 trials, one subject) slides. The median hybridization efficiency and the median percentage of metaphases with unexpected patterns of signal on the *A* and *B* slides were also without notable difference (Table II). Moreover, the low and indistinguishable percentages of metaphases on the *A* and *B* slides with only one SNRPN signal and the high and indistinguishable hybridization efficiencies derived from the *A* and *B* slides documented the reliability of the lot of probe.

Blind Study

Participants rejected similar proportions of the metaphases examined while attempting to score 50 metaphases on the *A* and *B* slides for determining hybridization efficiency (median = 7.4% and 8.7%, respectively). Participants in the blind study rejected larger proportions of the metaphases examined while attempting to score just 5 metaphases from either the 8 slides with a del(15)(q11.2→q12) or from the 15 with normal chromosomes 15 (median = 33.9 and 15.7%, respectively). Apparently, under the 2 sets of similar conditions, technologists applied differing criteria for scoring metaphases. This should be considered for FISH when developing and applying policies for becoming familiar with probes, for examining clinical samples and for recording workload.

There was no consistent problem with any of the 23 types of specimens provided by the participants (Table III). Participants reached a level of consensus $\geq$73.9% for the pattern of SNRPN signals in each of the 23 specimens. A pattern of SNRPN signals matching the cytogenetic standard was detected in 5 of 5 or 4 of 5 metaphases in 92.6% of the 529 challenges. A pattern of fluorescence matching the cytogenetic standard was found in $\geq$18 of 23 challenges by 20 laboratories and in $\leq$14 of 23 by 3 laboratories. Participants reported inconsistent, incomplete or no data for only 6.0% of the challenges, but half of such errors were made by just 2 of the 23 participants. Diagnostic errors were reported for 1.3% of the challenges, but the 7 errors were made

TABLE IV. Summary of Workload From 27 Timed Steps*

Steps	Labs	Mean	SD	Min	Max
Processing single slides					
Initial preparation	11	6:42	4:15	1:00	13:18
Hybridize	11	7:24	4:05	2:56	14:33
Posthybridization	11	3:27	2:38	1:33	10:24
Detection	11	2:22	0:54	1:10	3:37
Total	11	19:55	11:53	6:39	41:52
Processing batched slides					
Initial preparation	9	2:15	1:05	1:05	4:18
Hybridize	9	2:58	1:22	1:27	5:37
Posthybridization	9	1:22	0:53	0:45	2:54
Detection	9	1:42	0:48	0:45	3:22
Total	9	8:17	3:42	4:46	16:09
Analysis and documentation	11	27:29	16:35	5:37	55:20
Solution preparation	10	1:07	0:36	0:11	2:11

* Results expressed in minutes:seconds per slide.

by only 4 participants. Thus, the blind study identified laboratories needing improvement.

There was no apparent relationship between performance in the blind study and the type of microscope or system for detection of fluorescence used by participants. In general, however, participants reporting less total experience with FISH seemed more likely to make diagnostic and other errors in the blind study.

Workload Recording

Variation was evident in the complete and internally consistent sets of data from timings of the effort required for FISH (Table IV). Some of the apparent variation may have been due to inexperience with workload recording, inaccurate timings and incomplete directions for timing. Nevertheless, we believe that the remaining interlaboratory variations are real and do reflect different levels of efficiency.

From recording workload, processing slides one at a time required about 20 minutes, but processing 4 at a time only required about 8 minutes per slide. The time for analysis at the microscope, including capturing 2 documentary images per slide, averaged 27.5 minutes. Preparing solutions specifically for FISH consumed an additional 1.1 minutes for each specimen. With this information it is possible to calculate an average cost of labor associated with SNRPN and to estimate the number of technologists needed for studies with SNRPN.

Mosaicism in Prader-Willi Syndrome

The number of metaphases that need to be examined by FISH for clinical practice has not been established. The results of this project suggest that examining 5 metaphases with FISH for deletions may suffice when coupled with standard cytogenetics. Whenever mosaicism is suspected from clinical observations, conventional cytogenetics, or from metaphases with unexpected numbers of SNRPN signals, it would be important to examine more metaphases [Mowery-Rushton et al., 1994]. The number of additional metaphases could be determined statistically depending on the degree of confidence required, on the level of mosaicism to be excluded and on the efficiency of hybridization of the probe.

A single SNRPN signal appeared in 3.3% (54/1,617) of metaphases from the 15 patients with cytogenetically normal chromosomes 15. A single SNRPN signal also appeared in 3.4% (32/948) of metaphases collected from the *A* slides (21 different chromosomally normal patients) and in 3.2% (29/918) collected from the *B* slides (22 trials from one chromosomally normal patient) while determining hybridization efficiency of the SNRPN probe. We believe all these unexpected patterns of signals are artifacts of the testing system and are not indicative of mosaicism. Similarly, the 3.3% (27/821) of metaphases from the 8 patients with cytogenetically evident, nonmosaic del(15)(q11.2→q12) chromosomes showing 2 SNRPN signals are probably artifacts of the testing system. These observations suggest that detection of cell lines occurring at frequencies approaching 100% minus the hybridization efficiency (e.g., 3% for SNRPN) would be difficult to document. More examinations of metaphases from the same patients by multiple centers with extensive experience are needed to evaluate the problem of detecting mosaicism with FISH.

Future Direction

Results from the initial hybridization study showed no notable difference between slides prepared in individual laboratories and those prepared by a central laboratory. In the blind study, each of the 23 types of specimens was prepared by a different participant. Nevertheless, consensus was reached on all of the types of specimens at a level of ≥17 of 23 and participants experienced no consistent problem with any of the 23 types of specimens. Plainly, one or more laboratories could contribute slides to proficiency tests for FISH. This finding demonstrates the ease with which the pool of clinical challenges could be expanded for proficiency testing, clinical trials and evaluating guidelines for FISH.

The results from determining hybridization efficiency of the SNRPN probe and from the blind study

were summarized and distributed so that each participant could compare its result anonymously with those of other participants. This system of reporting is similar to the CAP/ACMG proficiency test for standard cytogenetics which has proven to be an excellent mechanism for improving laboratories [Hoeltge et al., 1993]. The CAP/ACMG proficiency test for standard cytogenetics expects that 80% of participants will report the same answer before a challenge is considered satisfactory. Since in the blind study 87% of the challenges were answered completely and in accord with the cytogenetic standard, our multicenter trial with molecular cytogenetics is comparable to the CAP/ACMG system. Further comparative and interlaboratory testing would help validate probes and improve both the experience and confidence of participants.

This study was not designed to establish the specificity and sensitivity of the SNRPN probe although such calculations could be made from the data in Table II. Nevertheless, we believe this initial work clearly demonstrates the feasibility of conducting a definitive study of the characteristics of SNRPN and other probes in multiple clinical laboratories using standardized instructions for application of a probe and uniform criteria for scoring fluorescent signals. Moreover, this project demonstrates that proficiency testing of FISH with an extensive array of challenges is possible and that multiple centers can collect data to evaluate workload. We believe this system, or a similar one, would improve the quality, efficiency and utility of FISH in clinical practice, just as proficiency testing has improved standard clinical cytogenetics.

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